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HCV Antibodies in Saliva and Urine

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Infection with hepatitis C virus (HCV) is usually established by detection of serum antibodies (anti-HCV). This study was conducted in order to evaluate whether saliva and urine may substitute serum for anti-HCV detection. Serum, saliva, and urine were obtained simultaneously from 141 patients with a variety of liver diseases and from 52 patients with autoimmune diseases (systemic lupus erythematosus n = 27 and rheumatoid arthritis n = 25). The cell free fraction of saliva and urine samples was tested for anti-HCV using a modification of a serum anti-HCV kit. Western blot analysis was used as a confirmation method. Of the patients with liver diseases, 73 were anti-HCV-seropositive. Salivary and urinary anti-HCV could be detected in 66 (90%) and 36 (49%) of the anti-HCV-serpositive patients, respectively. The presence of anti-HCV in saliva or urine was not related to the severity of liver disease. All the anti-HCV-seronegative liver patients were negative for salivary anti-HCV and 22 (32%) had urinary anti-HCV. The patients with autoimmune diseases were all anti-HCV-seronegative. None had detectable salivary anti-HCV while 33 (63%) were positive for urinary anti-HCV. Western Blot analysis confirmed the presence of anti-HCV in all serum and saliva samples tested but only in 2/12 urine samples. The results suggest that saliva, but not urine, may serve as a substitute for serum for the determination of anti-HCV positivity. ***J. Med. Virol. 55:24–27, 1998.***

KEY WORDS: serum; saliva; urine; anti-HCV

INTRODUCTION

Hepatitis C virus (HCV) is the main causative agent of non-A, non-B hepatitis [Choo et al., 1989; Kuo et al., 1989]. Infection with HCV is usually persistent and associated with chronic hepatitis, cirrhosis, and may progress to hepatocellular carcinoma [Rizzeto, 1992].

Routine diagnosis of HCV infection is based on detecting antibodies (anti-HCV) in serum [Alter et al., 1989]. Several reports indicate that antibodies to various viral infections can be detected not only in serum but also in other body fluids [Parry et al., 1987]. A correlation between serum antibodies and salivary or urinary antibodies has been reported in some viral infections, e.g., human immunodeficiency virus (HIV) [Archibald et al., 1986; Cao et al., 1989; Major et al., 1991; Crofts et al., 1991; Desai et al., 1991], hepatitis A virus (HAV) [Stuart et al., 1992; Parry et al., 1989], rubella [Saleh, 1991] and following immunization against polio virus [Zaman et al., 1991], rota virus [Ward et al., 1992; Friedman et al., 1993], and HAV [Hurni et al., 1993; Laufer et al., 1995]. As for HCV, oral fluid [Thieme et al., 1992; Sherman et al., 1994] and urine obtained from autopsy cases [Zhang et al., 1994] were suggested as alternative specimens for serum, for serodiagnosis of infection with this agent.

The present study was conducted in order to evaluate further the correlation between salivary and/or urinary HCV antibodies to serum HCV antibodies. For this purpose we tested, using a modified enzyme immunoassay (EIA), saliva and urine samples obtained simultaneously with serum samples from patients with various HCV-related and unrelated liver diseases and from patients with nonhepatic autoimmune diseases.

MATERIALS AND METHODS

Study Population

Two groups of patients were included in the study: group A,141 consecutive patients with a variety of liver

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diseases attending the Liver Clinic; and group B, 52 randomly chosen patients, with autoimmune diseases (27 with systemic lupus erythematosus (SLE), and 25 with rheumatoid arthritis), attending the Rheumatology Clinic, Soroka Medical Center, Beer-Sheva, Israel. Demographic and clinical data were available from the medical records of the patients. The study was approved by the institutional ethical board and an informed consent was obtained from all participants.

Sample Collection and Processing

Blood, saliva, and urine samples were obtained simultaneously from each patient visiting in the clinics during the years 1993–1996. Blood samples were collected in sterile tubes and centrifuged. Each serum sample was divided into two tubes which were kept in −70°C (for RNA determination) and in −20°C (for serology) until assayed. Midstream urine was collected in sterile containers. Saliva samples were obtained by asking the patient to spit into a sterile container. Both urine and saliva samples were centrifuged (1,600 rpm, for 20 min at 4°C). The cell free fraction was stored at −20°C until assayed.

Serum anti-HCV (IgG antibodies) were determined by a commercial kit: HCV EIA 2nd generation (Abbott laboratories, North Chicago, IL), according to the manufacturer's instructions, at a 1:40 dilution.

Determination of Salivary and Urinary Anti-HCV Antibodies

The urine and saliva samples were thawed, heat-inactivated (56°C for 30 min), and assayed using the same commercial kit used for serum. The assay was carried out according to the manufacturer's instructions except for sample concentration. Previous studies have shown [Parry et al., 1987] that the concentration of immunoglobulins in oral fluid is by far lower than serum. Preliminary studies in our laboratory have shown that the optimal conditions for anti-HCV determination were 1:2 dilution for saliva and undiluted for urine. Previous results from our laboratory have shown that pH adjustment had no effect on the sensitivity of anti-HIV detection in undiluted urine samples [Nitsan et al., 1994]. Therefore, pH adjustment was not performed.

The cutoff value for anti-HCV was calculated as follows:

$$\text{Cutoff value} = \text{XNC} + \text{XPC} \times 0.25.$$

Where XNC and XPC are the mean negative control and mean positive control optical densities (OD), respectively. For the determination of salivary XNC and XPC, 28 samples with the lowest OD (mean OD = 0.075) and 28 samples with the highest OD (mean OD = 1.720) were used. All the negative saliva samples were from patients negative for serum anti-HCV and all the positive samples were from patients positive for serum anti-HCV. For the determination of urinary XNC and XPC, 28 samples with the lowest OD (mean OD = 0.052) and 28 samples with the highest OD (mean OD = 0.980) were used. All the negative urine samples were from patients negative for serum anti-HCV and all the positive samples were from patients positive for serum anti-HCV. In each assay, positive and negative controls were run in triplicates.

TABLE I. Salivary and Urinary Anti-HCV in Relation to Serum Anti-HCV in Liver Clinic Patients

Liver clinic patients n = 141	Salivary anti-HCV (%)	Urinary anti-HCV (%)
Anti-HCV-seropositive n = 73	66 (90)	36 (49)
HCV-RNA-positive n = 65	60 (92)	33 (51)
Anti-HCV-seronegative n = 68	0 (0)	22 (32)

Confirmation of Anti-HCV Positivity

Western Blot (Innolia Innogenetics, Belgium) was used to confirm anti-HCV positivity. Serum samples were tested at 1:100 dilution according to the manufacturer's instructions. Saliva and urine samples were tested at a dilution of 1:10.

Detection of HCV-RNA

Total RNA was extracted from 200 μl of sera by QIAamp blood kit (QIAGEN GmbH, Germany) and eluted from the column with 50 ml of water containing 40 U of RNase Blocker I (Promega, Madison,WI). Reverse transcription (RT) of the 5′-noncoding region (NCR) of HCV-RNA was carried out with HCV antisense oligonucleotide (position 604-586) and 13 μl of RNA. The cDNA was amplified with Taq enzyme in polymerase chain reaction (PCR) using primers from positions 27-48 (5′ primer) and 348-329 (3′ primer). Confirmation of the PCR product was carried out by Southern blot hybridisation with a fourth primer (position 306-287).

HBsAg Determination

HBsAg was determined by a commercial kit (Abbott Diagnostics Laboratories) using an IMX apparatus, according to the manufacturer's instructions.

RESULTS

Of the Liver Clinic patients, 73 were anti-HCV-seropositive. Salivary and urinary anti-HCV were detected in 66 (90%) and 36 (49%), respectively (Table I). Sixty-eight patients were anti-HCV-seronegative. Of these, 38 were seropositive to hepatitis B surface antigen (HBsAg). All the anti-HCV-seronegative liver patients were negative for salivary anti-HCV and 22 (32%) had urinary anti-HCV.

All the anti-HCV-seropositive Liver Clinic patients (n = 73) were tested for HCV-RNA and 65 (89%) were found positive. Salivary anti-HCV was detected in 60/65 (92%) of the HCV-RNA-positive patients, and in 6/8

TABLE II. Western Blot Confirmation of Anti-HCV Determination

	Serum anti-HCV		Salivary anti-HCV		Urinary anti-HCV	
Patients	EIA[b]	WB[c]	EIA	WB	EIA	WB
1	+	+	–	–	+	–
2	+	+	–	–	–	NT[a]
3	+	+	–	–	–	NT
4	+	+	–	–	+	–
5	+	+	–	–	–	–
6	+	+	–	–	–	NT
7	+	+	–	–	–	NT
8	+	+	+	+	–	–
9	+	+	+	+	+	+[d]
10	+	+	+	+	–	–
11	+	+	+	+	+	+[e]
12	+	+	+	+	+	–
13	–	–	–	–	+	–
14	–	–	–	–	+	NT
15	–	–	–	–	+	–
16	–	–	–	–	+	NT
17	–	–	–	–	+	–
18	–	–	–	–	+	–

[a]NT, not tested.
[b]EIA, enzyme immunoassay.
[c]WB, Western blot.
[d]The urine sample was positive for antibodies to NS3 antigen. Antibodies to the following HCV antigens could be detected in this patient's serum and saliva, C, NS3, NS4 and C, NS3, respectively.
[e]The urine sample was positive for antibodies to NS3 antigen. The serum and the saliva of this patient were positive for antibodies to the following HCV antigens: C, NS3, NS4, NS5.

(75%) of the HCV-RNA- negative patients (P = n.s.). The clinical diagnosis of the liver clinic patients was: cirrhosis (n = 27), chronic hepatitis (n = 41), healthy carriers (n = 3), and acute hepatitis (n = 2). The presence of anti-HCV in saliva or urine was not related to clinical diagnosis.

The 52 patients from the Rheumatology Clinic were all anti-HCV-seronegative. None had detectable salivary anti-HCV, while a positive test for urinary anti-HCV was obtained in 33 (63%).

All the samples from anti-HCV-seropositive patients, negative for salivary anti-HCV (n = 7) were subjected to Western Blot (WB) analysis (Table II). In addition, 11 controls: 5 anti-HCV-positive in serum and saliva and 6 anti-HCV serum and saliva negative/urine-positive samples were tested by WB as well. This technique confirmed the results obtained by EIA in all serum and saliva samples. However, in urine samples, WB failed to confirm most of the EIA-positive anti-HCV results.

DISCUSSION

In the present study it was found that salivary anti-HCV had a 90% sensitivity and 100% specificity as a marker for serum anti-HCV. The detection of salivary anti-HCV was neither related to liver disease severity nor to presence of serum HCV-RNA. On the other hand, determination of urinary anti-HCV had a low sensitivity and a high rate of false positive results. Since a false positive anti-HCV result was reported when sera of patients with autoimmune disease were tested [McFarlane et al., 1990], we included a group of patients with rheumatoid arthritis or SLE. The results of anti-HCV testing in saliva and urine samples of these patients with autoimmune diseases confirmed the results of anti-HCV-seronegative patients with liver diseases. To the best of our knowledge, this is the only study evaluating the presence of anti-HCV in saliva, urine, and serum samples collected simultaneously from patients with a wide spectrum of diseases. The results suggest that saliva but not urine may serve as a substitute for serum for determing anti-HCV positivity.

Our results are in accord with two other previous studies. Thieme et al. [1992] reported on a 100% sensitivity and specificity of salivary anti-HCV in a small cohort of anti-HCV seropositive patients. A high sensitivity and specificity of salivary anti-HCV compared to serum was found by Sherman et al. [1994] as well. Their observation that the detection rate of salivary anti-HCV was not altered by the presence of current hepatitis B infection and inflammatory state of the liver, is in agreement with our results as well.

The use of oral fluid, which is a mixture of gingival crevicular fluid and saliva secretion, has been suggested as an alternative to serum antibody testing for HIV [Archibald et al., 1986; Cao et al., 1989; Crofts et al., 1991; Major et al., 1991], HAV [Stuart et al., 1992; Parry et al., 1989], and HBV [Ben-Aryeh et al., 1985], and shows promise. Although the salivary glands secretion contains mostly IgA, the crevicular fluid probably represents transudate of plasma, where IgM and IgG are predominant. Parry et al. [1987] noted that oral fluid samples would be expected to contain a range of IgG antibodies similar to serum, albeit at concentration 800- to 1,000-fold lower. Owing to the low concentration of immunoglobulins, we tested saliva samples at a dilution 20-fold lower than serum.

The results indicate that anti-HCV testing in urine is insensitive. Moreover, we found a high rate of positive anti-HCV results in urine samples of anti-HCV-seronegative patients. The correlation between EIA and Western Blot in urine samples was poor, suggesting that most EIA-positive results reflected a nonspecific false positive reaction. These results differ from those of Zhang et al. [1994] who reported an excellent correlation of anti-HCV detection between serum and urine. However, their study was carried out using serum and urine samples obtained from forensic autopsy cases and there is a possibility that urine samples were mixed with serum.

The usual method for detection of HCV infection is based on testing for HCV serum antibodies. Our results suggest that salivary HCV antibodies may have the potential of substituting HCV serum antibodies in immunodiagnosis. Nevertheless, the real issue before suggesting the clinical use of salivary anti-HCV testing is the positive and negative predictive value of the assay. The predictive value of a screening test is dependent on the prevalence of the disease in the popu-

lation tested, as well as on the sensitivity and specificity of the test [Mausner and Kramer,1985]. Therefore, taking in account the sensitivity and specificity for salivary anti-HCV determination, negative predictive values of 99.9%, 98.9% ,and 97.6% are obtained for populations with a prevalence of 1%, 10%, and 20% serum anti-HCV, respectively. The positive predictive value of this test is 100% regardless of the prevalence of serum anti-HCV in the population.

There are several advantages in using a simple saliva test. It is not only noninvasive and much simpler to use, but also protects the user from the ever-present danger of infection by exposure to needles. Saliva is easier to obtain than serum; its collection can be done by unskilled personnel and may be especially suitable for sampling newborns and young children and for Third World countries. The implementation of saliva would be of great economic value. It would save the cost of needles and syringes for obtaining blood and would save nurses' and physicians' time. Unlike previous studies [Thieme et al., 1992; Sherman et al., 1994], we did not use any special device for saliva collection, which makes sample collection even more simple and less expensive. Moreover, a positive salivary anti-HCV result should avoid withdrawing blood from a potential blood donor. Nevertheless, blood units from salivary anti-HCV-negative donors should be screened for serum anti-HCV.

In summary, the results suggest that saliva, but not urine, may serve as a substitute for serum in the determination of anti-HCV positivity. More studies and further improvement of the sensitivity of the method are needed.

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